Abstract

Background. Peripheral nerve injury-evoked neuropathic pain still remains a therapeutic challenge. Recent studies support the notion that progesterone, a neuroactive steroid, may offer a promising perspective in pain modulation.

Objectives. Evaluate the effect of progesterone administration on the development of neuropathic pain-associated allodynia and on the spinal expression of N-Methyl-D-Aspartate Receptor subunit 1 (NR1), its phosphorylated form (pNR1), and the gamma isoform of protein kinase C (PKCγ), all key players in the process of central sensitization, in animals subjected to a sciatic nerve constriction.

Methods. Male Sprague-Dawley rats were subjected to a sciatic nerve single ligature constriction and treated with daily subcutaneous injections of progesterone (16 mg/kg) or vehicle. The development of hindpaw mechanical and thermal allodynia was assessed using the von Frey and Choi tests, respectively. Twenty two days after injury, the number of neuronal profiles exhibiting NR1, pNR1, or PKCγ immunoreactivity was determined in the dorsal horn of the lumbar spinal cord.

Results. Injured animals receiving progesterone did not develop mechanical allodynia and showed a significantly lower number of painful responses to cold stimulation. In correlation with the observed attenuation of pain behaviors, progesterone administration significantly reduced the number of NR1, pNR1, and PKCγ immunoreactive neuronal profiles.

Conclusions. Our results show that progesterone prevents allodynia in a rat model of sciatic nerve constriction and reinforce its role as a potential treatment for neuropathic pain.

Key Words. Neuropathic Pain; Single Ligature Nerve Constriction; Neurosteroids; NMDA Receptor; NR1 Phosphorylation; Protein Kinase C Gamma

Introduction

Peripheral nerves are frequently exposed to mechanical trauma, metabolic disease, neurotoxic chemicals, infection or tumor invasion, which often trigger neuropathic pain [1]. This chronic pain condition is characterized by the presence of spontaneous and induced pain, such as hyperalgesia (increased pain perception of noxious stimuli) and allodynia (pain hypersensitivity to normally innocuous stimuli). Unfortunately, this maladaptive and unrelenting pain still remains difficult to alleviate.

Substantial evidence indicates that after nervous system injury, peripheral and/or central sensitization underlies neuropathic pain [1]. At the spinal cord level, central sensitization involves the increased expression and/or activity of the postsynaptic N-Methyl-D-Aspartate Receptor (NMDAR), a critical player for the development and maintenance of chronic pain [2,3]. All functional NMDAR contain an obligatory N-Methyl-D-Aspartate Receptor...
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subunit 1 (NR1) subunit, which is essential for receptor activity [4,5]. NMDAR function is critically modulated by post-translational modifications, including phosphorylation of NR1 on serine residues, a mechanism involved in receptor facilitation that is synchronized with the development of neuropathic pain behaviors [6–9]. Several conditions, like peripheral nerve injury [8], spinal cord trauma [9,10], diabetes [11,12], and morphine exposure [13] alter the expression and/or the phosphorylation of NMDAR subunits in the dorsal spinal cord, thus contributing to abnormal pain processing.

In addition, the gamma isoform of protein kinase C (PKCγ, [9,10], diabetes [11,12], and morphine exposure [13]) alter receptor facilitation that is synchronized with the development of neuropathic pain behaviors [6–9]. Several conditions, like peripheral nerve injury [8], spinal cord trauma [9,10], diabetes [11,12], and morphine exposure [13] alter the expression and/or the phosphorylation of NMDAR subunits in the dorsal spinal cord, thus contributing to abnormal pain processing.

A growing amount of literature supports the notion that neuroactive steroids display key regulatory effects in the control of pain and might represent a potential therapeutic approach for the treatment of neuropathic pain and the prevention of morphine tolerance [19–30]. Progesterone, in particular, mediates gestational antinociception [31], potentially contributes to sex-related differences in pain [31–33] and reduces pain sensitivity in intact rats [34].

Furthermore, progesterone and/or its reduced metabolites, significantly counteract the nerve dysfunction and axonal degeneration caused by neurotoxic chemotherapy [24,25,35], physical injury [19,23], aging [36], and diabetes [20], attenuating neuropathic pain-associated behaviors, although there is no general consensus [37].

Previous reports from our laboratory and others support the notion that progesterone also exerts neuroprotective effects in the central nervous system in animal models of neurodegenerative diseases and traumatic injuries [38–40]. More recently, we have shown that progesterone prevents allodynia after spinal cord injury and modulates the expression of several pain-related molecules with crucial roles in nociceptive processing at the spinal level [29].

Taken together, these previous data led us to hypothesize that progesterone, in addition to its neuroprotective effects on peripheral nerve fibers, could also prevent the injury-induced changes detected at the spinal cord level after nerve constriction. We addressed this issue by evaluating the effect of progesterone administration on the dorsal horn expression of NR1, the phosphorylated form of NR1 (pNR1), and PKCγ, all key molecules associated with central reorganization and pain-associated behaviors after peripheral nerve injury.

The present results show that progesterone prevents alldynia in a rat model of sciatic nerve constriction and reinforce its role as a potential approach for the treatment for neuropathic pain.

Methods

Sciatic Nerve Injury and Progesterone Administration

All experimental procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Assurance Certificate A5072-01 to Instituto de Biología y Medicina Experimental) and were approved by the local Animal Care and Use Committee. Care was taken to minimize animal discomfort and to limit the number of animals used. Male Sprague-Dawley rats (200–220 g), bred at the colony of the Instituto de Biología y Medicina Experimental, were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.). In a group of rats (N = 24), the right sciatic nerve was exposed and dissected free from the surrounding tissue at the mid-thigh level. It was then wrapped with a thin square strip (5 mm) of polyethylene and constricted with a tie around the strip using 3.0 silk suture (Barbour Threads, Lisburn, Ireland) to a “medium” single ligature nerve constriction (SLNC), with a reduction of 40–80% of its original diameter [41]. The degree of constriction of each nerve was confirmed after dissection under a surgery microscope using a 10 mm ruler, and also by microscopical observation of 16 μm sections stained with neutral red. In injured animals, the right sciatic nerve was constricted, while the left sciatic nerve was kept intact. Sham surgery was performed in another group of animals and consisted of exposing the right sciatic nerve in the same manner but without ligating the nerve.

Progesterone (Proluton, Schering Laboratories, Buenos Aires, Argentina; 16 mg/kg/day) was administered subcutaneously using oil as a vehicle, for a more gradual absorption, once a day, 3 hours before the behavioral tests and until the animals were euthanized. Injured animals received progesterone (16 mg/kg/day, N = 12), single ligature nerve constriction animals treated with progesterone (SLNC+PG) or vehicle (Ricine oil, Ewe, Sanitas, Argentina, N = 12, SLNC), starting immediately after performing the lesion. This dose of progesterone has been shown to prevent edema and neuronal loss and improve cognitive responses following brain contusion injury [39,42,43], and induce oligodendrogenesis and remyelination [44] and prevent mechanical and thermal alldynia [23] after spinal cord injury. As previously reported [45], the content of serum progestins was determined using a Coat-A-Count Progesterone RIA kit (Diagnostic Product Corporation, Los Angeles, CA, USA) at several time points during the treatment. Intra-assays and interassays coefficients of variation were 3.6% and 5.6%, respectively. Blood collection was performed on the same animals used for behavioral testing but on separate days. Control and injured animals receiving vehicle presented progesterin levels of 6.0 ± 1.2 and 6.6 ± 0.9 ng/mL, respectively. In animals chronically treated with progesterone, progesterin levels in the samples collected 3 hours before and 3 hours after the steroid injection were: 378 ± 56 ng/mL and 445 ± 36 ng/mL, respectively (P > 0.05), indicating that a steady level was achieved. Thus, the behavioral analysis was performed avoiding progesterone fluctuations or withdrawal. Further-
more, the selected dosage and the serum levels achieved are far below from those needed to produce anesthetic effects [46] or a dose-evoked sedation [47]. Different control groups were included: sham-operated rats (N = 16) receiving progesterone (N = 8) or vehicle (N = 8), as well as intact animals (N = 16) receiving progesterone (N = 8) or vehicle (N = 8).

**Behavioral Assessment**

Behavioral testing was performed during daytime (9:00–18:00) in all animals: 1 day before surgery, in order to obtain normal baseline values, and at different time points after sciatic nerve constriction or sham-operation, as previously described [29,48]. Briefly, the animals were placed in their acrylic testing chambers for 15 minutes for adaptation, and mechanical sensitivity was assessed with a series of 10 calibrated von Frey filaments (Stoelting, Woodale, IL, USA). The hairs were applied in ascending order (1, 2, 4, 6, 8, 10, 15, 26 g) onto the plantar surface of both ipsilateral and contralateral hindpaws [49]. Each hair was delivered three times with 5 seconds intervals. The lowest force at which application elicited a withdrawal response (brisk paw withdrawal together with a nocifensive behavior such as attack to the stimulus, escape responses or vocalization) was taken as the mechanical response threshold. An aversive paw withdrawal obtained with 6 g or less was considered an allodynic response. Cold sensitivity of the hindpaw to acetone (Choi test) was quantified by foot withdrawal frequency [50]. Thus, 100 µL of acetone was applied to the plantar surface of the paw using a plastic tubule connected to a 1 mL syringe. Acetone was applied five times to each paw at an interval of at least 5 minutes. The number of brisk foot withdrawals was recorded. Only rats showing normal responses to mechanical and thermal stimulation before surgery were included in the experiments. Behavioral testing was performed blindly and, as previously reported, results were analyzed using the Friedman Repeated Measures of Analysis of Variance followed by Multiple Comparison Test [29,48].

**Tissue Preparation**

Twenty two days after sciatic nerve constriction, the animals were deeply anesthetized with an overdose of chloral hydrate (800 mg/kg i.p.) and perfused through the heart with 60 mL of 0.9% NaCl, followed by 60 mL of fixative (4% paraformaldehyde in 0.16 M phosphate buffer, pH 7) at 4°C. Lumbar spinal segments (L4-L5) were removed and post-fixed in the same fixative for 90 minutes at 4°C. Tissues were then rinsed in 20% sucrose in phosphate buffer (pH 7.2) and stored in the same solution at 4°C.

**Immunofluorescence Procedure**

Tissues were embedded in OCT compound (Tissue Tek, Miles Laboratories, Elkhart, IN, USA) and cut transversally at 14 µm thickness in a cryostat (HM505N, Microm, Heidelberg, Germany). As previously reported [29], sections were mounted onto positively charged microscope slides, allowed to dry for at least 1 hour and rinsed twice in phosphate-buffered saline (PBS). After preincubation in 10% goat serum for 10 minutes at 37°C, sections were incubated overnight at 4°C with antibodies raised against PKCy (1:500, rabbit, Santa Cruz Biotechnology, Santa Cruz, CA, USA), NR1 (1:500, mouse, Upstate-Millipore, Billerica, MA, USA), or pNR1 (1:100, rabbit, Ser 896, Upstate-Millipore, Billerica, MA, USA), all diluted in PBS containing 2% goat serum and 0.2% Triton X-100. The sections were then rinsed twice in PBS containing 0.1% Triton X-100 and incubated for 1 hour at room temperature with goat anti-rabbit or goat anti-mouse secondary antibodies conjugated with fluorescein isothiocyanate (FITC) (1:200, Vector Laboratories, Burlingame, CA, USA). The sections were then rinsed three times in PBS and cover-slipped using Fluoromount G (Southern Biotech, Birmingham, AL, USA) as mounting media. In order to confirm the neuronal localization of the pNR1 subunit, cells were double labeled with an antibody directed against the specific neuronal marker neuronal nuclei (NeuN) (1:250, mouse, Upstate-Millipore, USA). Negative controls were prepared omitting the primary or secondary antibodies. Sections were examined under a Zeiss Axiosplan fluorescence microscope (Zeiss, Jena, Germany), and images were taken using a Nikon Eclipse E-800 confocal scanning laser microscope (Nikon, Tokyo, Japan).

**Quantification and Statistical Analysis**

The number of neuronal profiles exhibiting PKCy, NR1, or pNR1 immunoreactive (IR) signal was determined in the lumbar L4-L5 segments of the dorsal horn by counting immunostained profiles in randomly, systematically sampled sections throughout the spinal cord (every 10th section, 10 sections per spinal cord) [29]. Counting and data processing were performed by a blind observer. In order to determine the individual spinal cord laminae, the gray matter landmarks were first identified [51]. In the case of PKCy, almost all IR signal was located in lamina II, and the average number of PKCy-immunopositive neuronal profiles per section was determined for each animal. Data presented in the graph (Figure 3D) correspond to the mean ± standard error of the mean (SEM) of the number of PKCy immunolabeled profiles detected in each experimental group. On the contrary, NR1- and pNR1-IR profiles were observed throughout the dorsal horn and were therefore quantified in the following three regions: superficial dorsal horn (laminae I and II), nucleus proprius (laminae III and IV), and neck region (laminae V and VI), as previously described [9,29]. These regions were identified by cytoarchitectonic criteria [51], evaluating digital images visualized and analyzed with a computer-assisted image analysis system (Bioscan Optimas II software, Bioscan, Edwards, WA, USA) attached to the microscope [29]. The microscope illumination and data acquisition parameters were fixed throughout the entire analysis. The mean number of NR1- and pNR1-IR neuronal profiles per section was determined for each animal. These values were averaged within each experimental group and presented as group data. The mean area of each dorsal horn region was determined using the computer-assisted image analysis system, and
the results were therefore expressed as the mean number of NR1- or pNR1-IR profiles per unit area (1 mm²) [8,29]. The mean area of each of the dorsal horn regions evaluated did not change across the different experimental groups. In all the graphs, data show mean ± SEM. Statistical analysis was performed using GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, CA, USA) and carried out by applying the Kolmogorov–Smirnov normality test for Gaussian distribution and the Barlett’s Test for equal variances, followed by one-way analysis of variance (one variable: treatment, unmatched observations) and Newman–Keuls multiple comparison post-test. All control and experimental groups were included in this analysis. However, it should be noted that similar statistical differences were obtained when comparing each of the control groups with SLNC or SLNC+PG animals. Thus, only data corresponding to sham-operated animals receiving oil was presented in Figures 2 and 3.

Results

Behavioral Evaluation of Mechanical and Cold Alloodynia

All the control groups included in this study (see Methods) showed normal behavioral patterns when evaluated using the von Frey and Choi tests. No statistically significant differences were observed between any of these control groups at any of the time points evaluated (P > 0.05 when comparing any of the four control groups). Moreover, similar statistical differences were obtained when comparing each of the control groups with SLNC or SLNC+PG animals. Therefore, in order to facilitate the visualization of the results, only the behavioral assessment corresponding to sham-operated animals receiving oil (from now on referred as “control”, C) was included in the graphs (Figure 1A,B).

All the animals subjected to nerve constriction or sham-operation showed normal pain thresholds on both hindpaw footpads before surgery, and also on the contralateral hindpaw after surgery (Figure 1A,B). As previously reported [41,52], the single ligature constriction of the sciatic nerve induced the development of both mechanical and thermal allodynia in the ipsilateral hindpaw (SLNCi, Figure 1A,B). The withdrawal threshold to mechanical stimuli was significantly reduced 10 days after performing the lesion (P < 0.001 vs C, Figure 1A), and allodynic values were detected from day 14 until the animals were euthanized on day 22 (see Figure 1A). In addition, a significant increase in the number of painful responses to cold stimulation was detected from day 7 and maintained throughout the 3-week testing period (P < 0.001 vs C, from day 7 until day 22, Figure 1B). Interestingly, injured animals receiving progesterone (SLNC+PGi) showed significantly higher mechanical thresholds (P < 0.05 at day 10, P < 0.01 at day 14, P < 0.001 at days 17 and 21 vs SLNCi, Figure 1A), although they were not restored to normal levels (P < 0.01 at day 14, P < 0.05 at days 17 and 21 vs C, Figure 1A). However, treated animals did not develop mechanical alldynia and displayed a significantly lower number of positive responses to cold stimulation (P < 0.01 at days 7, 10, and 14, P < 0.001 at days 17 and 21 vs SLNCi, Figure 1B). In fact, the thermal threshold of these animals did not differ significantly from that detected in control rats (P > 0.05 vs C at all time points evaluated, Figure 1B). It should be noted...
NR1 and pNR1 Immunoreactivity

The number of NR1- and pNR1-IR neuronal profiles was not affected by sham-surgery or progesterone administration in control animals ($P > 0.05$ when comparing any of the four control groups, in each of the three dorsal horn areas evaluated, for both markers). As in the previous section, only data corresponding to sham-operated animals injected with vehicle (oil) was included in the figure as a control group (see Figure 2). However, as mentioned above, all control groups were included in the statistical analysis. As previously described [7,8,29], in control animals, both NR1- and pNR1-IR neuronal profiles were distributed throughout the three dorsal horn regions evaluated, with fewer cells in the superficial laminae and the majority of them found in deep laminae (V-VI). The progressive increase in the number of IR cells from superficial to deep laminae was more prominent in the case of pNR1, with almost a fivefold greater number of constitutive immunopositive cells detected in laminae V-VI in relation to those found in laminae I-II. As described in Methods section, the neuronal localization of the pNR1 subunit was verified by counting dorsal horn cells also exhibiting IR signal for the neuronal marker NeuN (Figure 4).

As in other models of neuropathic pain [8,29,53], the single ligature constriction of the sciatic nerve induced a dramatic increase in the number of NR1- and pNR1-immunopositive profiles in all the spinal cord regions evaluated ($P < 0.001$ vs C for both markers in the three regions; see SLNCi in Figure 2A–D). While the contralateral side remained unaffected ($P > 0.05$ vs C for both markers in the three regions), a significant increase in the total number of NR1-IR profiles was observed in the ipsilateral dorsal horn of animals subjected to the peripheral nerve injury (78% in laminae I-II, 67% in laminae III-IV, 53% in laminae V-VI), when compared with control animals. The raise observed in the number of pNR1-IR profiles was even more drastic: 308% in laminae I-II, 157% in laminae III-IV, and 147% in laminae V-VI.

Injured animals receiving progesterone presented a significantly lower number of neuronal profiles exhibiting NR1 immunoreactivity in the ipsilateral dorsal horn, when compared with that detected in SLNC animals ($P < 0.001$ in laminae I-II and III-IV, $P < 0.01$ in laminae V-VI vs SLNCi, Figure 2A,D). Thus, SLNC-induced increase in NR1-IR cells was attenuated 20–30% in animals receiving the steroid (28% in laminae I-II, 29% in laminae III-IV, 18% in laminae V-VI). Progesterone administration also resulted in a significantly lower number of pNR1-immunopositive profiles in the ipsilateral dorsal horn ($P < 0.001$ vs SLNCi in the three dorsal horn areas, Figure 2B,C), representing a 40–50% attenuation of the SLNC-induced increase: 54% in laminae I-II, 42% in laminae III-IV, 42% in laminae V-VI. The number of both NR1- and pNR1-IR profiles in the contralateral dorsal horn was not influenced by the lesion and/or progesterone treatment and remained similar to the values detected in control animals ($P > 0.05$ vs C for both markers in the three regions).

Although usually studied together [8,29], lamina V contains wide dynamic range neurons and lamina VI mainly proprioceptive neurons. Therefore, we have further analyzed the contribution of each of these laminae to the changes observed in the number of NR1- and pNR1-IR neuronal profiles in the different experimental groups. Nerve injury induced a significant increase in the number of NR1- and pNR1-IR neuronal profiles per unit area (62 ± 8% in lamina V and 40 ± 6% in lamina VI for NR1, $P < 0.001$ vs C for both laminae; 159 ± 20% in lamina V and 175 ± 21% in lamina VI for pNR1, $P < 0.001$ vs C for both laminae). Progesterone administration attenuated SLNC-induced increase in both laminae (20 ± 5% in lamina V and 14 ± 2% in lamina VI for NR1, $P < 0.01$ vs SLNC for both laminae; 42 ± 5% in lamina V, 42 ± 4% in lamina VI for pNR1, $P < 0.001$ vs SLNC for both laminae). Therefore, both laminae independently contributed to the changes in NR1 and pNR1 immunoreactivity detected after injury and progesterone administration.

PKCγ Immunoreactivity

As previously described [54,55], in control animals, PKCγ IR signal was detected in a dense band of fibers and small neurons located in the inner region of lamina II (Figure 3A). Similarly to what has been observed in other animal models of neuropathic pain [15,29], SLNC rats showed significantly higher numbers of PKCγ-immunopositive neuronal profiles in the ipsilateral dorsal horn ($P < 0.001$ vs C, see SLNCi in Figure 3B,D), 22 days after performing the lesion. With the exception of a few cells in lamina III, the IR signal remained restricted to lamina III (Figure 3B). Interestingly, progesterone administration attenuated this increase, and thus a significantly lower number of neuronal profiles exhibiting PKCγ immunoreactivity could be detected in the ipsilateral dorsal horn ($P < 0.05$ vs SLNCi, see SLNCi+PGi in Figure 3C,D). The contralateral side remained unaffected in both SLNC and SLNC+PG animals ($P > 0.05$ vs C in both cases).

No statistically significant changes were observed when comparing the number of PKCγ-IR neuronal profiles in the different control groups ($P > 0.05$ in all cases). Thus, sham surgery and/or progesterone administration did not affect PKCγ IR pattern in control animals. Moreover, similar statistical differences were obtained when comparing each of the control groups with SLNC or SLNC+PG animals. Therefore, only data corresponding to sham-operated animals injected with oil were presented in Figure 3.

Discussion

To our knowledge, the present report is the first to show that progesterone administration was able to prevent the injury-induced increase in the number of NR1- and PKCγ-IR neuronal profiles in the ipsilateral dorsal horn of animals subjected to a SLNC. Moreover, progesterone...
administration also resulted in a significantly lower number of neurons displaying pNR1 immunoreactivity. In accordance with the proposed role of these molecules in the dorsal horn pain circuit, the steroid also prevented the development of neuropathic pain-associated allodynia.

Activation of spinal dorsal horn NMDAR is essential for the development of central sensitization, phenomenon that underlies the development of neuropathic pain-associated behaviors such as allodynia [2,3]. Furthermore, phosphorylation of NMDAR NR1 subunit via several protein kinases has been recognized as a major mechanism involved in the regulation of NMDAR function leading to receptor facilitation and contributing to central sensitization in several chronic pain conditions [6–8,56] and also related to the development of morphine tolerance [13].
Figure 2 Graphs (A,B) and photomicrographs (C,D) illustrating the effect of peripheral nerve single ligation constriction and progesterone administration on the number of NR1-IR (A,D) and pNR1-IR (B,C) neuronal profiles in the ipsilateral dorsal horn. The number of both NR1- and pNR1-IR neurons per unit area (1 mm²) was significantly increased in animals subjected to the nerve lesion (see SLNCi). Progesterone administration dramatically attenuated the injury-induced increase in both NR1- and pNR1-IR cells in the three dorsal horn areas evaluated (SLNC+PGi). In (A,B) values show mean ± SEM and were analyzed applying one-way analysis of variance (ANOVA) and Newman–Keuls multiple comparison post-test. The following symbols were used to represent P values: ns P > 0.05; * P < 0.05; ** P < 0.01, and *** P < 0.001. In (C,D), representative photomicrographs showing pNR1 (c) and NR1 (d) immunoreactivity in the ipsilateral dorsal horn of the lumbar (L4-L5) spinal cord from animals corresponding to the different experimental groups. Note that NR1-IR neuronal profiles are clearly visualized in high magnification images (D). Calibration bar: 350 μm in (C), 90 μm in (D). Control = sham-operated animals receiving oil; SLNC = single ligation nerve constriction animals receiving oil; SLNC+PG = single ligation nerve constriction animals treated with progesterone; i = ipsilateral dorsal horn. NR1 = N-Methyl-D-Aspartate Receptor subunit 1; IR = immunoreactive; pNR1 = phosphorylated form N-Methyl-D-Aspartate Receptor subunit 1; ns = not significant.

In naive rats, constitutive pNR1 immunoreactivity was found in cells of the deep dorsal horn but minimally detected in the superficial layers (I–II). In agreement with other reports [7,8,53,56], we found that the number of pNR1-IR neurons significantly increased throughout the entire dorsal horn (both superficial and deep layers) in animals subjected to the sciatic nerve chronic constriction. As previously reported, this widespread NR1 subunit phosphorylation probably suggests that many of these dorsal horn neurons acquiring NMDAR activation receive C-afferent inputs directly and/or indirectly and that some of them may be projection neurons that convey nociceptive information to higher levels of the central nervous system [53,56]. Interestingly, progesterone was able to attenuate the injury induction of pNR1-IR profiles in the three dorsal horn areas evaluated, a result that correlates with the reduction of both thermal and mechanical allodynia.

However, the mechanical thresholds were not fully restored to normal levels during progesterone administration. This could be related to the fact that the expression of the molecules evaluated was not completely restored to control values during the steroid treatment. Nevertheless, it should be stressed that the responses to mechanical stimuli were significantly attenuated in animals receiving progesterone as compared with untreated injured animals and definitively not allodynic. Furthermore, the thermal threshold of animals treated with progesterone did not differ significantly from that detected in control rats.

Moreover, injured animals receiving progesterone also displayed a decreased number of PKCy-IR neuronal profiles. PKCy, an intracellular signaling kinase, is expressed by a subset of excitatory interneurons located in the inner region of lamina II [54,55] and displays a critical role as a mediator of neuropathic pain [54]. Activation of PKC potentiates the phosphorylation of NMDAR [17,57] and contributes to amplify the NMDAR-dependent spinal circuit underlying persistent pain [17,18,57].

Progesterone, by preventing the injury-induced increase in spinal NR1-, pNR1-, and PKCy-IR neuronal profiles may inhibit NMDAR-mediated effects on nociception, and therefore provides a valuable tool for alleviating allodynia after peripheral nerve injury. However, the mechanisms responsible for these analgesic effects of progesterone remain to be determined.

Extensive data in the literature support the idea that progesterone is much more than a reproductive hormone [38,40,58]. In the nervous system, progesterone exerts pleiotropic effects by regulating gene transcription, intracellular signaling pathways, and neurotransmission [38,40,59]. The spinal cord is a target for progesterone actions as it expresses several progesterone receptors, including the classic nuclear receptor (PR), a ligand-activated transcription factor that regulates the expression of target genes [38,40]. The observed effects of progesterone administration on NR1 and PKCy expression may probably involve transcriptional regulation by direct and/or indirect activation through progesterone receptors [29]. In this regard, the newly cloned progesterone membrane receptors [60] and the progesterone receptor membrane compound 1, PGRMC1 [61], localized in dorsal horn neurons, also offer probable sites for progesterone actions in pain mechanisms. However, other multiple receptor sites and mechanisms could not be precluded when evaluating these results.

The sigma-1 receptor (Sig-1R), strongly expressed in the dorsal spinal cord [62] and associated with central sensitization and pain [63], is also a target for progesterone [64,65]. Recent findings demonstrate that spinal Sig-1R activation enhances NMDAR-induced pain via PKC-dependent phosphorylation of the NR1 subunit [66,67]. This observation is consistent with our results and...
Figure 3 Photomicrographs (A–C) and graph (D) showing the number of neuronal profiles exhibiting PKCγ immunoreactivity in the ipsilateral dorsal horn of the lumbar (L4-L5) spinal cord from animals that were subjected to sham operation (Control), nerve ligation (SLNCi) or nerve ligation, and progesterone administration (SLNCi+PGi). Boxed areas in (A–C) are shown at a higher magnification in the corresponding inset. In the three experimental groups, PKCγ immunoreactive signal was highly restricted to a subset of neuronal profiles mainly confined to the inner region of lamina II (A–C). As it can be observed, the dramatic increase in the overall number of PKCγ-IR cells observed after the lesion (B,D) was attenuated by progesterone treatment (C,D). In the graph, values show mean ± SEM and represent the average number of immunoreactive profiles per section. One-way ANOVA followed by Newman–Keuls post-hoc test was performed for statistical comparisons and the following symbols were used to represent P values: * P < 0.05; ** P < 0.01, and *** P < 0.001. Calibration bar: 50 μm in (A–C). Control = sham-operated rats receiving oil; SLNC = single ligature nerve constriction animals receiving oil; SLNC+PG = single ligature nerve constriction animals treated with progesterone; i = ipsilateral dorsal horn; IR = immunoreactive; PKCγ = gamma isoform of protein kinase C; SEM = standard error of the mean.
suggests that progesterone, acting as a competitive inhibitor of Sig-1R [64,65], may reduce the number of neurons expressing the pNR1 subunit in a crucial area for nociceptive processing.

In addition, reactive oxygen species (ROS) have recently been shown to play an important role in neuropathic and capsaicin-induced pain, predominantly through spinal mechanisms [56]. ROS enhance NR1 phosphorylation through the activation of protein kinases C and A [56], and spinal nerve ligation-induced phosphorylation of the NR1 subunit in dorsal horn neurons is attenuated by ROS scavengers [68]. Furthermore, ROS are critical factors involved in nerve injury-induced spinal microglia activation and proinflammatory cytokines production, processes that participate in the initiation of neuropathic pain [68]. Relevant to our work, progesterone has been shown to reduce endogenous oxidative stress, decreasing free radical production and lipid peroxidation [58,69] and to inhibit the synthesis of inflammatory mediators and glial activation [39]. Therefore, the attenuation of pNR1 induction observed in animals treated with progesterone could also be explained, at least partially, through this mechanism.

Other important steroid-mediated actions may derive from the conversion of progesterone into its reduced metabolites 5α-dihydroprogesterone and 3α, 5α-tetrahydroprogesterone, also called allopregnanolone. Both peripheral nerves and spinal cord are active centers able to synthesize and metabolize progesterone [38,40]. Furthermore, the endogenous progesterone production and/or metabolism is modulated both in the peripheral nerve [26] and the spinal cord [22,70] after injury. More importantly, the local enhancement of the reductive activity of 3α-hydroxysteroid oxido-reductase, which catalyzes the interconversion of the reduced derivatives, has been shown to exert a pivotal role in the modulation of spinal sensory networks after sciatic nerve injury [21]. Reduced metabolites may mediate progesterone-induced analgesia by modulating the gamma-aminobutyric acid (GABA_\text{A}) receptor complex and T-type calcium channels [34,71–73] or by enhancing the expression of specific GABA_\text{A} receptor subunits [74]. Several studies sustain this idea, showing that the hormone exerts anxiolytic and anesthetic effects through its metabolism to allopregnanolone [46,75]. Interestingly, a recent clinical study demonstrated an inverse association between endogenous allopregnanolone serum levels and self-reported pain, suggesting an antinociceptive role for this neurosteroid in human pain conditions [76].

Several other studies have also shown that the administration of progesterone and/or its reduced metabolites significantly counteracts biochemical, morphological, and functional alterations of injured nerves [19,20,23–25,35,77]. In particular, these neuroactive steroids restore nerve conduction velocity, thermal thresholds, and skin innervation density and improve nerve Na,K-ATPase activity and the expression of myelin proteins in experimental models of peripheral neuropathy [19,20]. Recent studies have also revealed that endogenously produced neurosteroids modulate dorsal root ganglia sensory function [78] and protect dorsal root ganglia neurons against injury-induced apoptosis [79], further supporting their protective and regenerative effects that contribute to attenuate neuropathic pain-associated behaviors [24–26].

Thus, successful repair and functional preservation of nerve axons and dorsal root ganglia cells are closely related to the alleviation of pain behaviors. Moreover, peripheral nerve
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injury also induces plastic changes in the spinal circuitry that are related to the emergence of neuropathic pain [1]. Interestingly, in addition to restoring cellular and functional alterations in peripheral nerve fibers and dorsal root ganglia, progesterone is also able to prevent the spinal maladaptive changes involving dorsal horn NMDAR activation after peripheral nerve constriction.

The manifestation of chronic pain also depends on descending facilitatory and/or inhibitory pathways arising from supraspinal sites [80]. Recent reports also suggest that activated glial cells both at spinal [81] and supraspinal levels [82] may modulate neuronal activity and contribute to persistent pain. In this regard, progesterone-linked changes in glial activation [83,84] and descending systems arising from supraspinal sites [85] may contribute to modulate spinal nociceptive processing. Thus, it is likely that the convergence of multiple mechanisms at supraspinal, spinal, and peripheral sites may underlie the neurochemical changes observed in the dorsal horn after progesterone administration.

These findings extend our previous observations [29] demonstrating that progesterone favors a molecular environment at the dorsal horn level that contributes to prevent allodynia after spinal cord injury.

As aforementioned, damage to peripheral nerves stimulates several maladaptive responses in the sensory nervous system, which involve multiple mechanisms, including ectopic generation of action potentials, facilitation and disinhibition of synaptic transmission, aberrant synaptic connections, and neuroimmune interactions [1]. For this reason, recent reports strengthen the value of new therapeutic strategies designed to prevent the multiple cellular and molecular events that underlie the maladaptive plasticity within the nociceptive system [1]. In this regard, progesterone emerges as an ideal molecule to achieve this goal. Progesterone, an easy-to-administer agent, by targeting multiple mechanisms and working on a variety of receptors may represent a reliable drug to attenuate the deleterious cascade associated with peripheral or central nervous system damage [58]. In fact, two recent independent phase II clinical trials support the neuroprotective effects of natural progesterone as a valuable molecule for the treatment of acute traumatic brain injury [58]. Furthermore, two phase III clinical trials, which are currently performed, will provide the final evidence of progesterone’s safety and efficacy [58].

In summary, the present results provide new compelling data to further support progesterone-based strategies for the treatment of neuropathic pain after peripheral or central nervous system insults.

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